

Priority Report

Erythropoietin Activates Cell Survival Pathways in Breast Cancer Stem-like Cells to Protect Them from Chemotherapy

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Abstract

Recombinant erythropoietin (EPO) analogs [erythropoiesis-stimulating agents (ESA)] are clinically used to treat anemia in patients with cancer receiving chemotherapy. After clinical trials reporting increased adverse events and/or reduced survival in ESA-treated patients, concerns have been raised about the potential role of ESAs in promoting tumor progression, possibly through tumor cell stimulation. However, evidence is lacking on the ability of EPO to directly affect cancer stem-like cells, which are thought to be responsible for tumor progression and relapse. We found that breast cancer stem-like cells (BCSC) isolated from patient tumors express the EPO receptor and respond to EPO treatment with increased proliferation and self-renewal. Importantly, EPO stimulation increased BCSC resistance to chemotherapeutic agents and activated cellular pathways responsible for survival and drug resistance. Specifically, the Akt and ERK pathways were activated in BCSC at early time points following EPO treatment, whereas Bcl-xL levels increased at later times. *In vivo*, EPO administration counteracted the effects of chemotherapeutic agents on BCSC-derived orthotopic tumor xenografts and promoted metastatic progression both in the presence and in the absence of chemotherapy treatment. Altogether, these results indicate that EPO acts directly on BCSC by activating specific survival pathways, resulting in BCSC protection from chemotherapy and enhanced tumor progression. *Cancer Res*; 73(21): 6393–400. ©2013 AACR.

Introduction

Erythropoiesis-stimulating agents (ESA) have been used for two decades in the supportive therapy of patients with cancer, due to their ability to increase red blood cell production and to reduce the need of transfusions (1). In 2002, ESAs were administered to approximately 45% of all patients with cancer (2). However, following clinical trials reporting a shorter progression-free survival and/or overall survival in ESA-treated patients, ESAs were suspected to increase the risks of thromboembolic events and to enhance tumor progression (3–6). Consequently, in 2008, the U.S. Food and Drug Administration (FDA) limited the indication for ESA administration to patients with cancer with hemoglobin levels of less than 10 g/dL receiving chemotherapy for palliative intent (7). Since then, the use of ESAs in patients with cancer declined progressively,

and recently the FDA released new guidelines ensuring that ESAs' access is strictly controlled and that patients are fully informed about ESA-related risks (8). Despite clinical observations suggesting a possible association between ESAs and tumor progression, the effect of erythropoietin (EPO) on neoplastic cells remains a matter of debate. In particular, experimental studies on the effect of EPO on cancer cells yielded controversial results, likely due to variable methodologic approaches. Recent *in vivo* studies, however, provided important clues on tumors' response to EPO. Specifically, EPO was shown to antagonize the effect of trastuzumab on breast cancer xenografts and to decrease the effect of chemotherapy in a mouse model of metastatic breast cancer (9, 10). Such studies suggest a direct influence of EPO on breast tumors and highlight the importance of reliable *in vivo* models to elucidate the interactions between EPO and tumor cells. The existence of cancer stem cells (CSC) in solid tumors was shown for the first time in breast cancer, in which CSCs were isolated as a CD44⁺/CD24^{−/low} population able to initiate tumors with as few as 200 cells (11). Lately, breast tumorigenic cells were identified either by distinctive phenotypes such as ALDH⁺, CD24^{high}/CD49f^{high}/delta-like 1 (DLL1)^{high}, CD24^{high}/CD49f^{high}/Delta-notch like EGF repeat-containing transmembrane (DNER)^{high}, or through functional characteristics such as enhanced PKH26 dye-retaining capacity or low proteasome activity (reviewed in ref. 12). More recently, breast CSCs (BCSC) were identified as a ganglioside GD2⁺ population able to form tumors with as few as 10 cells (13). BCSCs have been shown to increase after chemotherapy treatment (14) and to be quantitatively

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associated with chemotherapy resistance (15). Moreover, BCSCs have been shown to mediate invasion and metastasis both *in vitro* and in mouse models (16). Elucidating the effect of EPO on BCSC is therefore crucial to fully understand the effects of ESAs treatment in patients with breast cancer. As ESAs administration is reserved to patients with metastatic breast cancer receiving chemotherapy, it is particularly important to understand whether they may influence BCSC response to anticancer drugs and metastasis progression. Here, we used human BCSC-derived orthotopic/metastatic xenografts to show that BCSC response to EPO *in vivo* results in increased chemotherapy resistance of primary tumors and metastases, resulting in enhanced tumor progression.

Materials and Methods

Antibodies and reagents

Primary antibodies were: mouse monoclonal anti-EPOR MAB307 (R&D Systems; ref. 10), rabbit polyclonal anti-EPOR M20 (Santa Cruz Biotechnology; ref. 9), rabbit polyclonal anti-Akt and rabbit polyclonal anti-phospho-Akt Ser 473 (9272 and 9271; Cell Signaling Technology), mouse monoclonal anti-phospho Erk1/2 Tyr 204 (E4; Santa Cruz Biotechnology), rabbit polyclonal anti-Erk1 (K23; Santa Cruz Biotechnology), mouse monoclonal anti-Bcl-xL (H-5; Santa Cruz Biotechnology), mouse monoclonal anti- β -actin (JLA20; Calbiochem), mouse monoclonal anti-CD44 (BU75; Ancell), mouse monoclonal anti-CD24 (HIS50; BD Biosciences), mouse monoclonal anti-p63 (4A4; Santa Cruz Biotechnology), mouse monoclonal CK8-18 (5D3), mouse monoclonal CK14 (LL002), and mouse monoclonal CK5 (XM26; all from Novocastra), mouse monoclonal anti-Ki67 (MIB-1; Dako), mouse monoclonal anti-CD49f (MP4F10; R&D Systems). Secondary antibodies were: horse-radish peroxidase (HRP)-conjugated anti-mouse antibody (Pierce), HRP-conjugated anti-rabbit antibody (Thermo Scientific), mouse fluorescein isothiocyanate (FITC)- and Rhodamine red-conjugated antibodies (Invitrogen, Molecular Probes), and mouse R-phycoerythrin (PE) antibody (Sigma-Aldrich). Recombinant human EPO was purchased from R&D Systems.

BCSC isolation and culture

Human breast cancer tissues were obtained from patients undergoing surgery in accordance with the ethical standards of the institutional Committee on human experimentation (authorization no. CE-ISS 09/282). Tumor tissues were mechanically and enzymatically digested with collagenase (1.5 mg/mL; Gibco) and hyaluronidase (20 mg/mL; Sigma-Aldrich) in Dulbecco's Modified Eagle Medium (Gibco), shaking for 1 hour at 37°C. The resulting cell suspension was plated in ultra-low attachment flasks (Corning) in serum-free medium supplemented with basic fibroblast growth factor (bFGF; 10 ng/mL) and EGF (20 ng/mL) as previously described (17). This procedure yielded BCSC lines that were subjected to genotyping to validate each cell line individuality and were further tested for their ability to generate tumor xenografts that replicated the histology of the parental tumor.

Viability, proliferation, and clonogenic assays

For viability assays, BCSCs untreated or pretreated 24 hours with 3 U/mL EPO were cultured for the indicated times in presence of doxorubicin (1 μ mol/L), 5-FU (5-fluorouracil; 25 μ mol/L), or Taxol (5 μ mol/L). The number of viable cells was detected by the CellTiter Aqueous Assay Kit (Promega). Cell death was also assessed by acridine orange (50 μ g/mL)/ethidium bromide (1 μ g/mL) staining and fluorescence microscopy detection. Colony-forming assays were conducted on soft agar (Seaplaque) with 0.4% base agar and 0.3% top-layer agar. After 21 days, colonies were stained with 0.01% crystal violet and visualized under a light microscope.

Immunoblotting

Cells were growth factor-starved for 24 hours and treated with 3 U/mL EPO for 10, 30, 120 minutes and 48 hours. Protein extracts were obtained in ice-cold T-PER buffer (Thermo Scientific) with protease inhibitors (Sigma-Aldrich). Equal amounts of proteins were loaded on SDS-PAGE gels and transferred to nitrocellulose membranes, subsequently blocked with 5% nonfat dry milk in TBS containing 0.1% Tween 20 and probed with primary and HRP-linked secondary antibodies. Immunoreactive bands were visualized with Super-Signal West Dura Substrate (Pierce). Image acquisition was conducted with a ChemiDoc Imaging system (UVP Advanced Imaging Systems).

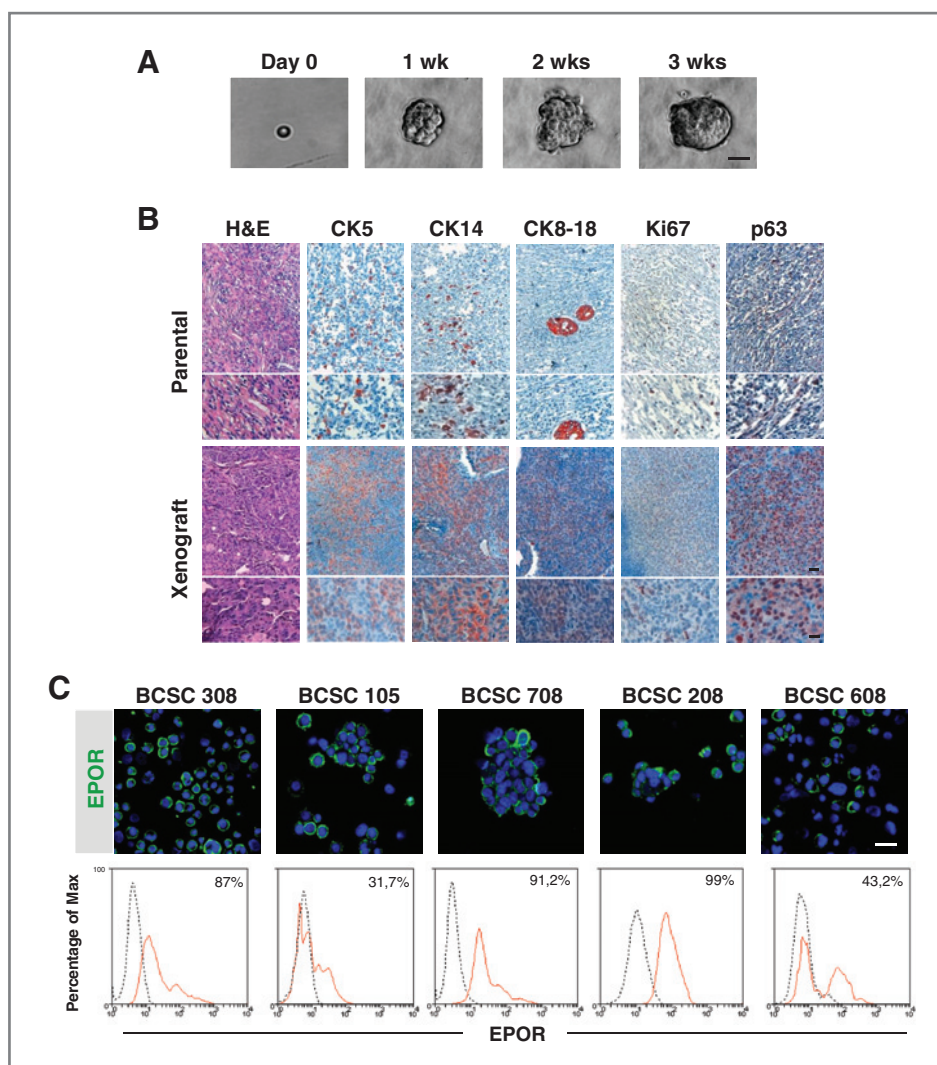
Immunohistochemistry

Apoptotic cells on paraffin-embedded breast cancer xenograft sections were visualized by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction with the *In Situ* AP Cell Death Detection Kit (Roche). Immunohistochemical analyses were conducted on 5 μ m-thick paraffin-embedded sections of breast cancer tissue and xenografts. Tissues were heated for antigen retrieval and stained with specific antibodies against Bcl-xL, CK 8-18, CK 14, CK 5, p63, Ki67, EPO receptor (EPOR), or isotype-matched controls overnight at 4°C. Sections were incubated with biotinylated anti-mouse or anti-rabbit immunoglobulins and subjected to streptavidin-peroxidase (Dako). Stainings were revealed using 3-amino-9-ethylcarbazole substrate (AEC; Dako) substrate and cells counterstained with aqueous hematoxylin. Slides were mounted with synthetic resin.

Immunofluorescence and flow cytometry

Immunofluorescence was conducted on cytopins of cultured BCSC fixed with 2% paraformaldehyde for 20 minutes at 37°C, blocked with 0.5% bovine serum albumin for 30 minutes and exposed overnight at 4°C to antibodies against EPOR, CD44, and CD24. Stained slides were treated with Rhodamine Red- or FITC-conjugated anti-mouse antibodies with the addition of 200 ng/mL RNase (Sigma-Aldrich). Nuclei were counterstained with TOTO-3 iodide (Invitrogen-Molecular Probes) and images were acquired using an Olympus FV1000 confocal microscope. For fluorescence-activated cell sorting (FACS) staining, BCSC were fixed with 2% paraformaldehyde and stained with primary antibodies against CD44, CD24, EPOR, or isotype-matched controls and

Figure 1. Characterization of BCSC lines and BCSC-derived xenografts. **A**, microscopic imaging of progressive sphere formation by a single BCSC (line 308). Bar, 25 μ m. **B**, immunohistochemical staining with hematoxylin and eosin (H&E) or antibodies against cytokeratins 5 (CK5), 14 (CK14), 8–18 (CK8–18), Ki67, and p63 of sections derived from breast carcinoma (Parental) and from a mouse xenograft (Xenograft) generated with BCSC derived from the same tumor (BCSC line 308). Bar, 40 μ m (inset, 30 μ m). **C**, EPO receptor expression on BCSC lines detected by fluorescence microscopy (top; bar, 25 μ m) or flow cytometry (bottom).



then with fluorochrome-conjugated secondary antibodies. Samples were analyzed with a FACSCalibur equipped with CellQuest Software (BD Biosciences).

Mice treatment

Animal studies were carried out according to the institutional guidelines under the Italian Ministry of Health authorization (DM 23/2011-B). BCSCs (3×10^5) were suspended in 100 μ l of 1:6 Matrigel (BD Biosciences) and orthotopically injected in 5-week-old nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (Charles River Laboratories). Tumor size was measured weekly with an electronic caliper and volume was calculated using the formula: $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$. After 4 weeks, mice were treated intraperitoneally either with doxorubicin (2 mg/kg, on day 2 and 5 every week for 4 weeks) or 5-FU (150 mg/kg, on day 1 every week for 4 weeks), alone or in combination with EPO (150 U/kg, on day 1 and 4 every week for 4 weeks). PBS was used as control. At the end of the treatment, mice were sacrificed and tumors were collected for histologic analyses. To determine the

in vivo effects of EPO on a metastatic breast cancer model, NOD/SCID mice were orthotopically injected with 4.5×10^5 BCSCs carrying a Tween Luciferase–GFP lentiviral vector. After cell inoculation, mice received a subcutaneous injection of D-luciferin (150 mg/kg; Promega) and were analyzed by *in vivo* imaging (Biospace Laboratories). Five weeks later, primary tumors of mice showing lung metastases (as measured by luciferase intensity) were removed and mice ($n = 4$ per group) received respectively intraperitoneal injections of PBS, paclitaxel (10 mg/kg, on day 1 every week for 3 weeks), EPO (300 U/kg on day 2 every week for 3 weeks), or paclitaxel + EPO. Three weeks later, mice were euthanized and lungs were analyzed for luciferase expression. Data were quantified with Biospace Lab M3 Vision software.

Statistical analysis

Data were expressed as mean \pm SD. The statistical significance of results was determined by Bonferroni multiple comparison tests. Results were considered significant when *P* values were less than 0.05 (*, *P* < 0.05; **, *P* < 0.01; and ***, *P* < 0.001).

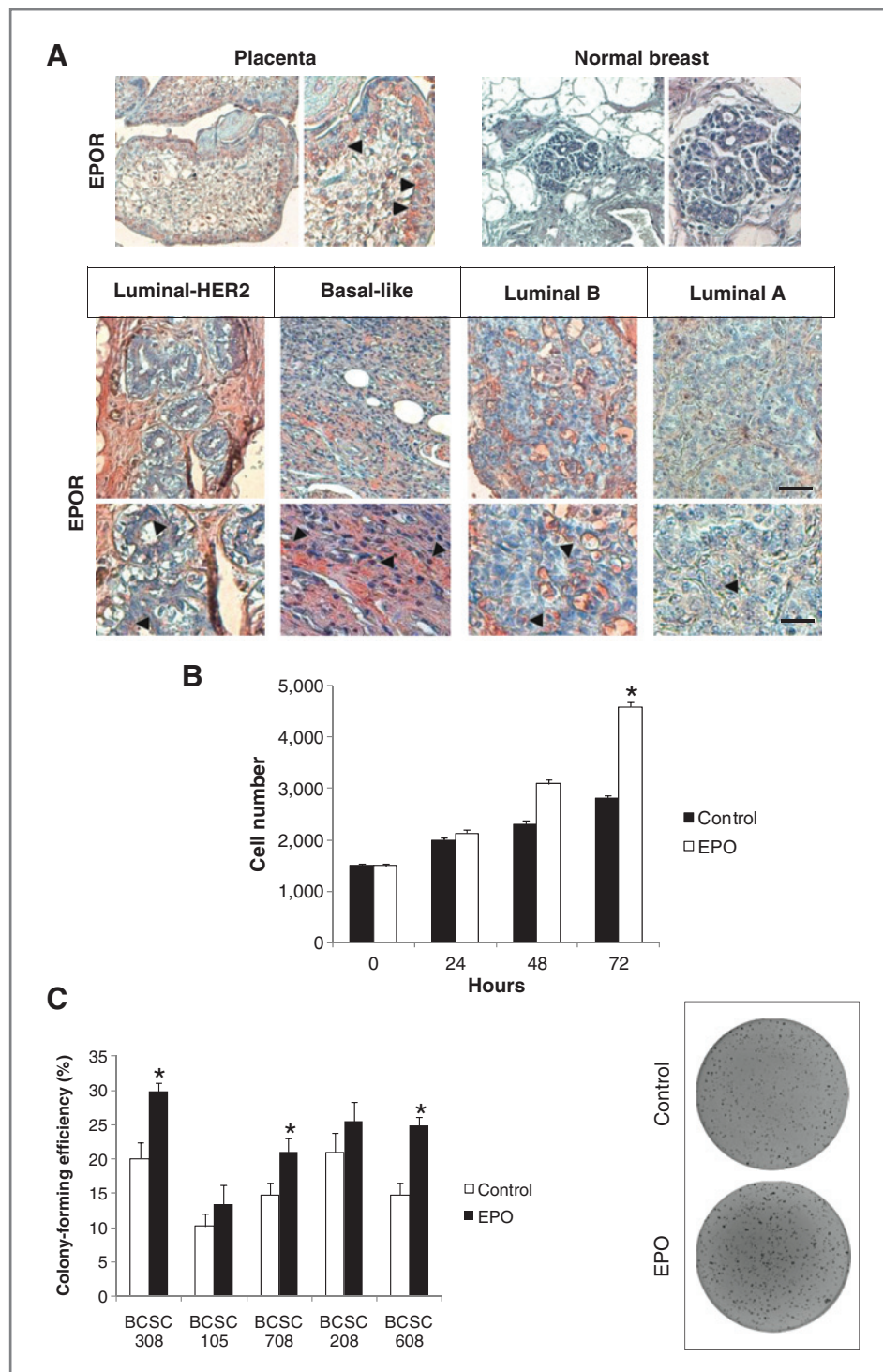


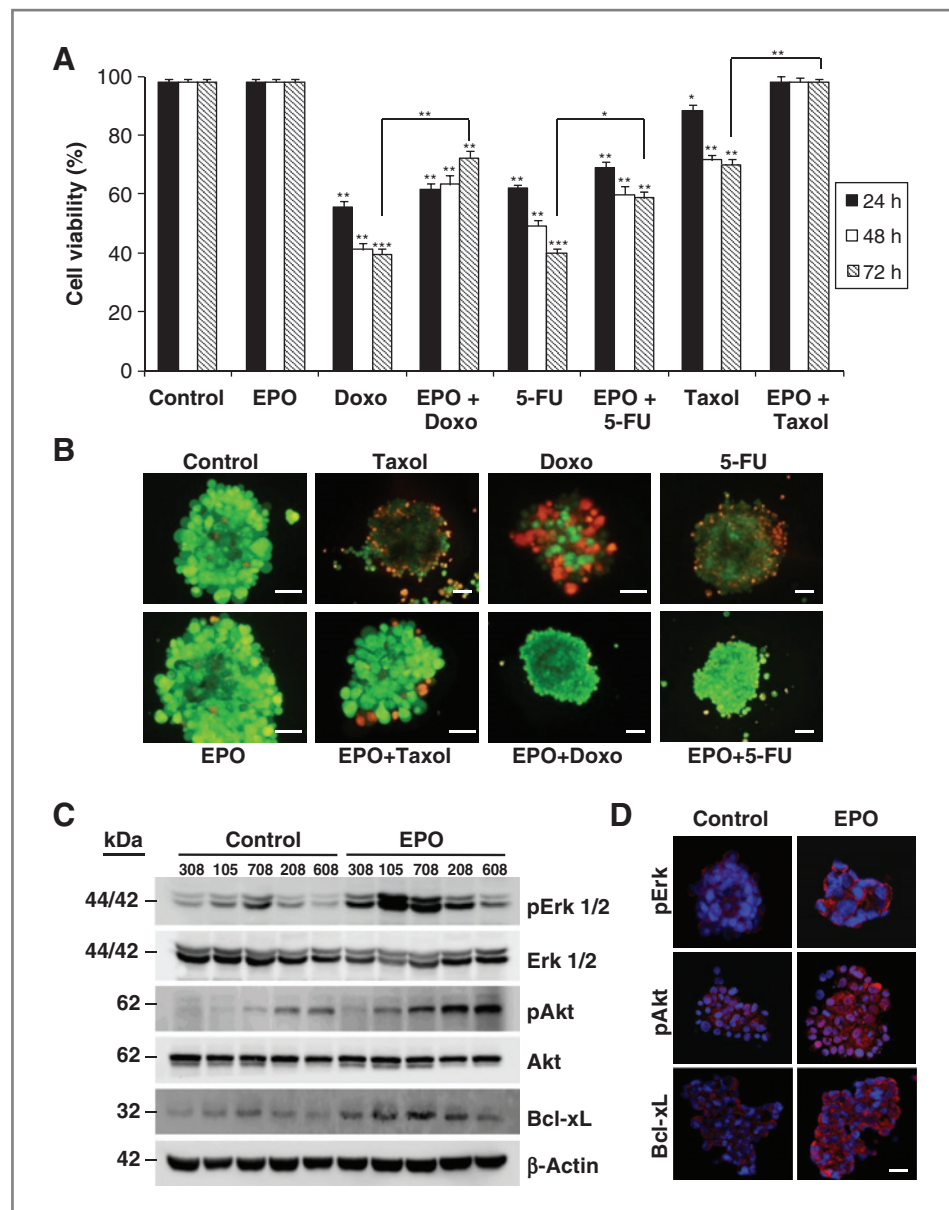
Figure 2. EPOR expression in breast cancer subtypes and EPO response of cultured BCSC. **A**, EPOR staining of tissue sections derived from human placenta, normal breast (top), and breast tumors of different subtypes (bottom). Black arrows indicate EPOR-positive cells. Bar, 45 μ m (inset, 25 μ m). **B**, number of cells obtained after 72 hours of culture in the absence (control) or in the presence of EPO 3 U/mL (EPO). Results shown are the mean \pm SD of experiments carried out in triplicate with five BCSC lines. *, $P < 0.05$. **C**, number of colonies generated in semisolid culture conditions by BCSC lines in the absence (Control) or in the presence (EPO) of EPO 3 U/mL (left). Representative picture of the plates (BCSC line 308; right). *, $P < 0.05$.

Results and Discussion

BCSCs can be isolated from tumor specimens by selective culture in medium containing EGF and bFGF (18), resulting in a majority of CD44⁺/CD24^{-low} cells that form progressively expanding tumor spheres (Fig. 1A and Supplementary Fig. S1A). According to this method, five BCSC lines were isolated from infiltrating breast tumors (Supplementary Table S1). Cells

isolated in such conditions fulfilled the functional characteristics of CSCs, as they were able to produce tumors in immunocompromised mice that replicate the original patient tumor in terms of histologic structure and marker expression (Fig. 1B). As a first step to investigate the potential BCSC sensitivity to EPO, we assessed whether the EPO receptor was detectable on cultured CSC and on tissue sections of different breast

Figure 3. EPO increases BCSC resistance to chemotherapy *in vitro* by stimulating cell survival pathways. **A**, BCSC untreated (Control), treated with EPO 3 U/mL (EPO), with chemotherapeutic agents doxorubicin (Doxo; 1 μ mol/L), 5-FU (25 μ mol/L), or Taxol (5 μ mol/L) and with the combination EPO-chemotherapy were assessed for cell viability after 24, 48, and 72 hours. Results shown are the mean \pm SD of experiments carried out in triplicate with five BCSC lines. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **B**, ethidium bromide/acridine orange staining of tumor spheres (BCSC line 308) treated as above. **C**, levels of phospho-Erk (pErk), phospho-Akt (pAkt), and Bcl-xL in BCSC untreated (Control) or growth factor-starved and stimulated with 3 U/mL EPO, respectively, for 10 minutes, 2, and 48 hours. **D**, cells treated as in C (BCSC line 208) were stained with the indicated antibodies and visualized by fluorescence microscopy. Bar, 25 μ m.



tumor subtypes, whose BCSC content was reportedly related to increasing malignancy (19). Because of previous controversies about the specificity of anti-EPO receptor antibodies, we used only antibodies that were validated by recent authoritative studies (see Materials and Methods). The specificity of the anti-EPO receptor antibody used for immunofluorescence and flow cytometry was further validated by assessing its ability to detect EPO receptor increase in leukemic UT-7 EPO cells upon growth factor starvation (Supplementary Fig. S1B; ref. 20). Staining of intact BCSC with anti-EPOR antibody revealed substantial (31–99%) EPOR expression on all the BCSC lines examined (Fig. 1C), indicating a potential role of EPO in the regulation of BCSC proliferation and survival. EPO receptor was also detected on cultured BCSCs stained with CD49f antibodies (Supplementary Fig. S1C), indicating its presence

on BCSC populations identified with different stem cell-associated markers. In tissue sections, strong EPO receptor expression was detected on the positive control (placenta) and on basal-like tumors. EPO receptor was also present, at a lesser extent, on HER2⁺ and luminal B tumors, whereas a faint expression was detectable on luminal A tumors and on normal breast, the latter showing a positivity at the limit of detection (Fig. 2A and Supplementary Table S2). Treatment of BCSC cultures with recombinant human EPO resulted in increased cell proliferation, indicating that EPOR expressed on BCSC surface is functional and delivers signals that modulate cell growth (Fig. 2B). To determine whether EPO was able to affect BCSC self-renewal, cells derived from dissociated mammospheres were plated in soft agar in the presence or in the absence of EPO. Colony scoring after 3 weeks showed a

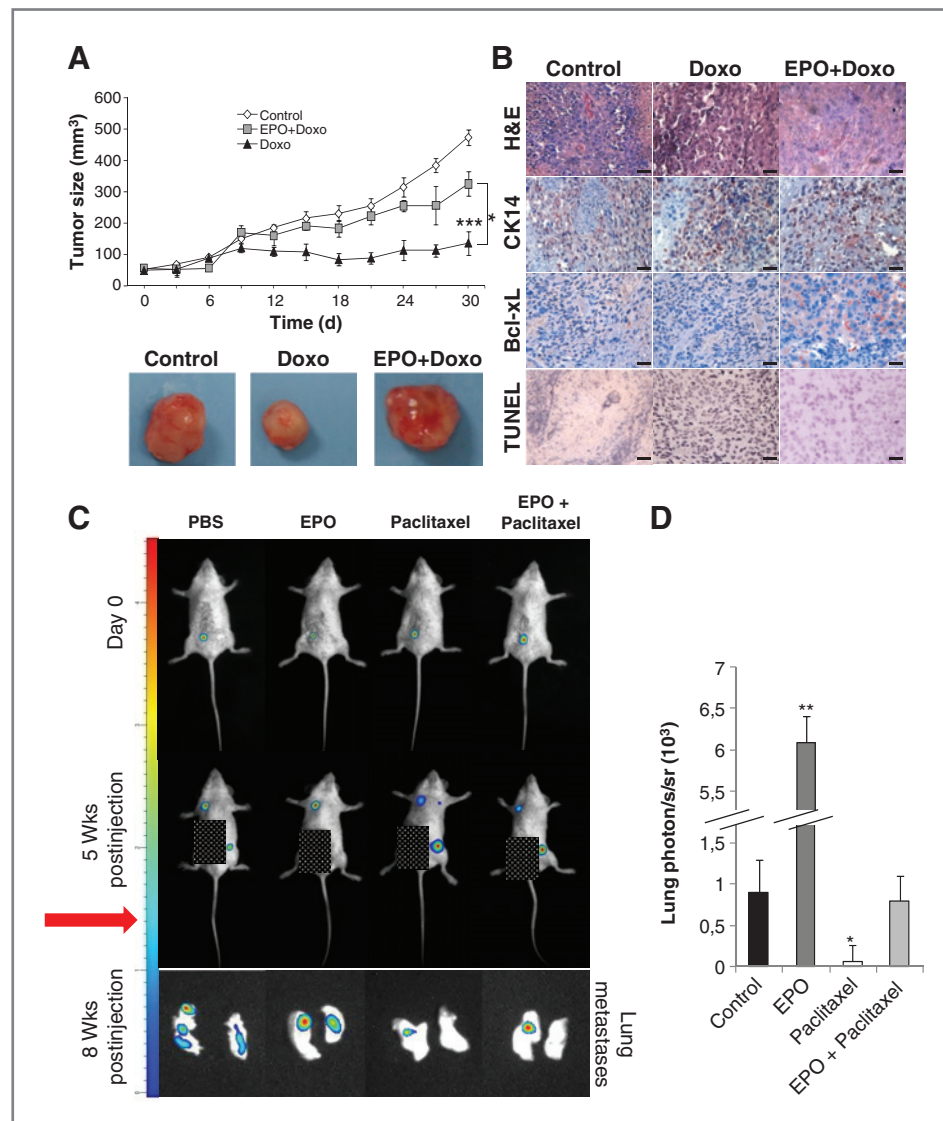


Figure 4. EPO protects primary and metastatic tumors from chemotherapy *in vivo*. **A**, growth of BCSC-derived tumor xenografts (BCSC line 308) vehicle-treated (Control), treated with doxorubicin (Doxo) alone, or in combination with EPO (Doxo+EPO) as described in Materials and Methods (top). Results shown are the mean \pm SD of three experiments carried out with groups of 3 mice each. *, $P < 0.05$; ***, $P < 0.001$. Representative pictures of the tumors (bottom). **B**, immunohistochemical staining of xenograft sections obtained at the end of the experiment shown in **A** and stained with hematoxylin/eosin (H&E), with anti-cytokeratin 14 (CK14), anti-Bcl-xL (Bcl-xL), or TUNEL. Bar, 30 μ m. **C**, whole-body imaging of tumors at different time points after injection (Day 0) of 5×10^5 BCSCs transduced with Tween-LUC GFP in the mammary fat pad of NOD/SCID mice, as described in Materials and Methods. Five weeks after injection, when lung metastases (and in some cases peritoneal metastases) were visible, the primary tumor was removed and the treatment with EPO, paclitaxel, or paclitaxel + EPO was started (red arrow). After 3 weeks of treatment (8 weeks postinjection), mice were sacrificed and lungs were subjected to bioimaging to detect metastatic tumors (Lung metastases). The black square on the left side of the mice was positioned to shield luciferase signals emitted from residual cells that remained after primary-tumor removal. One representative experiment of 4 mice per group is shown. **D**, photon counts emitted from mice lungs derived in the experiment described in **C**. Photon/s/sr, photons per second per steradian. *, $P < 0.05$ and **, $P < 0.01$.

significantly higher number of colonies in EPO-treated samples in 3 of 5 cases, indicating that EPO can increase BCSC self-renewal *in vitro* (Fig. 2C).

BCSCs were previously shown to be more resistant than bulk tumor cells to chemotherapeutic drugs (14). Because EPO is used almost exclusively in patients with cancer undergoing chemotherapy, it is essential to clarify whether EPO can further enhance BCSC resistance to chemotherapy, therefore favoring drug resistance and tumor relapse. Upon treatment of mam-

mospheres with EPO and with chemotherapeutic agents commonly used for breast cancer therapy, we found that the presence of EPO resulted in increased BCSC survival in the presence of cytotoxic drugs (Fig. 3A and B). This observation indicates that EPO activates survival signals in BCSCs that are responsible for chemoresistance. To identify pathways downstream of EPOR that may be responsible for apoptosis resistance in BCSC, we analyzed levels of phospho-Akt, phospho-Erk, and Bcl-xL at different time points upon EPO stimulation

(Fig. 3C and Supplementary Fig. S2). Phosphorylation/activation of Erk and Akt was maximal respectively 10 minutes and 2 hours after EPO stimulation in 5 of 5 and 4 of 5 BCSC lines (Fig. 3C). Differently, an increase in Bcl-xL levels was not apparent at early time points of EPO stimulation (Supplementary Fig. S2), but became clear in 5 of 5 BCSC lines after 48 hours of treatment (Fig. 3C). Increase in pErk, pAkt, and Bcl-xL upon EPO stimulation was apparent also in intact spheres (Fig. 3D).

To investigate whether EPO treatment affected tumor response to chemotherapy *in vivo*, we produced orthotopic breast tumors by injecting BCSC in the mammary fat pad of NOD/SCID mice. Tumors were allowed to grow until they reached the size of 50 mm³, then mice were treated with EPO and/or chemotherapeutic agents doxorubicin (Fig. 4A and B) or 5-FU (Supplementary Fig. S3A and S3B) for 4 weeks, during which tumor volume was constantly monitored with an electronic caliper. Although vehicle-treated tumors grew exponentially, chemotherapy-treated tumors were significantly inhibited. In contrast, the growth of tumors treated with EPO + chemotherapy was similar to controls, indicating a chemoprotective effect of EPO *in vivo* (Fig. 4A and B and Supplementary Fig. S3A). Staining of xenograft sections at the end of the treatment revealed an increased rate of apoptosis and lower levels of Bcl-xL expression in chemotherapy-treated tumors but not in tumors treated with chemotherapy and EPO in combination (Fig. 4B and Supplementary Fig. S3B). These results indicate that EPO reduces the efficacy of chemotherapy *in vivo* by promoting BCSC apoptosis resistance. To investigate whether EPO could influence the growth of metastatic tumors, we injected luciferase-transduced BCSC in the mammary fat pad of NOD/SCID mice and awaited the formation of spontaneous lung metastases. Five weeks after BCSC injection, when metastases started to be detectable, the primary tumor was removed to observe the effect of subsequent treatments solely on metastatic sites. Mice were then treated for 3 weeks with EPO alone, with paclitaxel, or with the EPO + paclitaxel combination. At the end of the treatment, mice were sacrificed and lungs were analyzed for luciferase expression. Tumor burden in lungs of mice treated with EPO alone was strongly increased as compared with that of control mice (Fig. 4C and D). Enhanced metastatic progression was also found in the lungs of mice treated with the paclitaxel + EPO combination as compared with mice treated with pacli-

taxel alone, indicating that EPO exerted a chemoprotective effect on metastatic tumors (Fig. 4C and D).

Because the disclosure of clinical trials showing that ESAs treatment had an adverse influence on patient survival, the effect of EPO on tumor cells has been the subject of an intense debate. Although ESAs likely influence patient survival through multiple mechanisms, few *in vivo* studies have specifically addressed the question of whether EPO modifies tumor response to therapy. We have shown for the first time that EPO can bind and stimulate BCSCs, resulting in increased tumor growth and chemoresistance. These results confirm and expand previous observations by Hedley and colleagues on xenografts obtained with breast cancer cell lines (10). In patients with breast cancer, EPO-mediated BCSC stimulation may not result in immediate effects on tumor growth or response to chemotherapy, as BCSCs represent a minority of cells, but may favor subsequent tumor relapse. Further clinical studies that evaluate rates of relapse in ESA-treated patients would be required to clarify this issue.

Disclosure of Potential Conflicts of Interest

R. De Maria is a consultant/advisory board member of AACR. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: M. Todaro, G. Stassi, A. Zeuner

Development of methodology: M. Todaro, A. Turdo, R. Dattilo, G. Stassi, G. Federici

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Todaro, A. Turdo, M. Bartucci, F. Iovino, M. Biffoni

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Bartucci

Writing, review, and/or revision of the manuscript: M. Biffoni, A. Zeuner

Study supervision: M. Todaro, G. Stassi, R. De Maria, A. Zeuner

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